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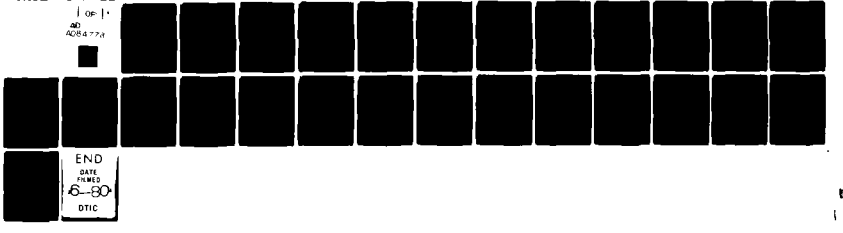

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PNEUMONITIS OF POST TRAUMATIC AND SEPTIC STATES: IDENTIFICATION OF CAUSATIVE AGENTS

Annual Report

GEORGE H. A. Clowes, Jr., M.D.

1 April 1977

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

In a continuing effort to isolate the circulating factor or factors responsible for the development of adult respiratory distress syndrome after trauma or the onset of sepsis, the plasmas of 40 patients were tested in a perfused rabbit lung bioassay. Of these, plasma from 60% of the post traumatic patients and 89% of the septic patients caused edema and an elevation of pulmonary artery pressure in the test lung. Of 12 pigs with peritonitis

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induced by cecal ligation, 6 gave evidence of a pulmonary shunt by the presence of hypoxemia. Plasma from the same animals were positive by bioassay. The administration of heparin or trasylol to the animals caused the test to become negative.

The presence of one or more abnormal peptides of molecular weight 4000-6000 in the plasma of septic patients was demonstrated by Amicon filtration, column chromatography, and thin layer chromatography. A rabbit antibody was made to this fraction of plasma from septic patients which only reacted with septic plasma after absorbtion with normal plasma. Heating the plasma to 56°C inactivated the response. By immunoelectrophoresis both the antibody and rabbit anti C3 gave similar responses. It is concluded that a peptide associated with activation of the clotting cascade or with the presence of inflammation, possibly C3A, may be responsible for initiation of "shock lung" or "septic lung".

INTRODUCTION

The present progress report concerns the results of clinical observations and experimental studies directed at an understanding of the factors which induce pneumonitis in post traumatic and seriously infected patients. Previous progress reports (1975) and published papers (1) (3) (4) have presented evidence for the existance of agents with low molecular weights injurious to the lung which circulate in the plasma of such patients or experimental animals with sepsis. Experiments in which a rabbit lung was perfused with non-blood containing solutions suggested that bacterial endotoxin does not directly affect the lung. On the other hand, using such a preparation fractions of plasma from seriously injured or infected patients containing substances of molecular weights ranging from 1000 to 10000, when introduced into the perfusate, caused edema and a rise of pulmonary vascular resistance in the test lung (1) (7). A variety of observations have indicated that these substances are peptides (5).

The research of the past year has been directed toward amplification of clinical observations on the adult respiratory distress syndrome. At the same time, attention has been directed toward identification of the circulating agents which induce interstitial pneumonitis (6) (Phase I) and the typical secondary bronchopneumonia (Phase II) which still carries a high mortality in the seriously injured or septic patient. To pursue this work a bioassay, molecular filtration, chromatography, and immunological techniques have been employed. The results of these studies are presented in this report.

I. CLINICAL OBSERVATIONS

During the past two years the pulmonary function of 40 additional traumatized and septic patients has been studied in the surgical intensive care units of the Boston City Hospital. The values obtained are presented in Table 1. They confirm observations previously made upon post-shock and septic patients by the principle investigator and his colleagues (3) (4). In essence it is demonstrated that trauma and hypovolemia, followed by resuscitation and surgery cause a three fold increase in pulmonary shunting. Dynamic compliance is reduced nearly 50% as shown by the elevation of peak inspiratory pressures. In this group pulmonary vascular resistance and wedge pressure (left atrial) remain relatively unchanged. This state of pulmonary dysfunction seldom was prolonged beyond four days unless sepsis occurred. On the other hand, in patients who became seriously infected the process was prolonged, especially when bronchopneumonia developed following the initial interstitial pneumonitis. In the septic group pulmonary artery pressures rose, and evidence of some degree of left heart failure was evident from wedge pressures which averaged approximately twice those of normal people at rest. Shunting was 25% greater than in the post traumatic group without infection and required more than 3 times longer respiratory support.

II. PERFUSED LUNG BIOASSAY

A preparation for perfusion of rabbits 1.5Kg body weight, was described in detail (progress report 1975). In essence, the animals are anesthetized with pentobarbital. A trachial tube is placed through a trachiostomy, and connected to a Harvard respirator. Through a midline sternal splitting incision the pulmonary artery is cannulated through the right ventricle. The pulmonary venous system is drained through a cannula in the apex of the left ventricle. After ligation of the pulmonary artery around this cannula and occlusion of the aorta, perfusion is instituted to wash the blood from the lungs. Subsequently the perfusion is continued with a non-blood perfusate containing Ringer lactate and a colloid solution adjusted to maintain an osmotic value equivalent to that of the animal plasma. To assure the absence of sympathetic reflexes a cervical spine column section is carried out.

The circuit consists of large bore polyvinal tube leading from the left heart to the reservoir which is a plastic transfusion bag or polyethelene cylinder. From the reservoir the perfusate is pumped by a rotary pump through a silicone rubber tube to the pulmonary artery cannula. The volume in the perfusate circuit is approximately 100 ml. The reservoir is immersed in a water bath to maintain perfusate temperature at 35 to 37°C. Carbon dioxide is bubbled through the reservoir at a rate sufficient yo maintain the pH of the solution between 7.3 and 7.4. The reservoir is placed 15 cm below the left heart to be certain that positive pressure does not exist in the left atrium. Pressure in the

pulmonary artery is recorded continuously on a polygraph through a transducer attached to a small tube which passes through the cannula. The tip lies beyond the cannula in the lumen of the pulmonary artery. Ventilation of the lungs with air is continued at a volume adequate to fill but not distend them. Insufflation pressure is continuously recorded through the side arm in the endotrachial tube. By this means the presence of edema or bronchial constriction is shown by an increase of peak inspiratory pressure (PIP). The perfusion rate is approximately 100 ml per Kg of rabbit weight per minute. A pressure of 15 to 20 cm H_2O is established. The perfusion is allowed to continue for 45 minutes to be certain that no artefactual reaction of edema or pulmonary vascular constriction occurs. The sample of plasma (3ml) or plasma fraction (0.5ml) to be tested is then injected into the reservoir. A positive reaction is demonstrated within 15 to 20 minutes by a rise of pulmonary artery pressure to 50% or more above the baseline, edema formation and the presence of bubbles in the endotrachial tube. When no reaction occurs following introduction of the test substance into the circuit, histamine (0.3mg) is injected into the reservoir to be certian that the preparation is reactive.

During the past year the perfusion fluid consisted of 5% gelatin in Ringer lactate solution buffered to pH 7.4. Following the performance of 102 test perfusions the supply of gelatin was exhausted. The replacement gelatin proved to be inadequate for this preparation. If 5% gelatin was used edema frequently occurred

before a test could be carried out. This suggested that a substance was present in the gelatin which caused a reaction in the lung. Since that time numerous experiments have been carried out employing various samples of gelatin and other substances to establish an adequate osmotic pressure. None have proven satisfactory until recently. Within the last six weeks a series of perfusions employing Ringer lactate with 2% albumin and 3% polyvinyl pyrolidine have worked well. All of this time has been required for standardization.

Prior to failure of the bioassay system satisfactory tests were completed on the plasma of 25 patients who are included among those presented in Table 1. The results of the assay are given in Table 2. All but one of the plasma specimens from the septic patients induced edema and a rise of pulmonary edema with a loss of compliance in the rabbit lung. A positive reaction occurred with 60% of the five plasmas following hypovolemic shock. The two plasmas which failed to react after haemorrhage were drawn from patients who bled from the gastro-intestinal tract but were not injured. Unfortunately, the numbers in this series were inadequate for these differences to be statistically different.

Tests employing the bioassay were successful in 66 other experiments in which studies were carried out. On the plasma of 12 pigs rendered septic by cecal ligation and given endotoxin (3mgm/ by body weight), six of the septic animals developed hypoxemia on the second day postoperative. The plasma from each of these six animals produced pulmonary edema in the rabbit lung. Only

two of the other six reacted. No difference was found between the activity of pulmonary venous blood and arterial blood, suggesting that the active agent in the plasma was not emmanating from the lung. All but three of the animals given endotoxin died. Seven gave evidence of pulmonary shunting. Plasma from five of these animals gave a reaction by bioassay. The bioassay was negative in the other five.

Three days following the induction of peritonitis, Heparin, 500 units per kilogram of body weight, was given to four animals with positively reacting plasma. Four hours later when blood from these heparinized animals was drawn, the plasmas of two remained reactive in the bioassay, and two were negative. On the fourth day the plasma of each was reactive again in the bioassay. Administration of Trasylol, 1,000,000 units*, intravenously during a four hour period, caused the plasma of three to become non-reactive in the bioassay. These findings strongly suggest that a polypeptide is being activated in the septic animals. Since heparin seems to have a blocking effect it is possible that the production of fibrinopeptides is being inhibited. On the other hand, it is well known that both heparin and trasylol are non-specific peptidase inhibitors in high concentration. Therefore, it is possible that the production of other active peptides such as the kinins (2) or compliment, may be inhibited. These data support the observations of Voss et. al. (5) in our laboratory some years ago in which transfer of a circulating agent in dogs was blocked by trasylol therapy.

*Supplied by Bayer Corp., Wuppertal, W. Germany.

III. STUDIES ON ISOLATION AND IDENTIFICATION OF ABNORMAL FACTORS IN SEPTIC PLASMA.

A. Chromatography

1.) Column: (Sephadex G-50): Heparinized plasma from normal, septic and post traumatic patients is fractionated by cold pressure filtration into three parts containing substances of different molecular weights: a.) 0-1000 molecular weight, b.) 1000-10000 molecular weight, and c.) greater than 10000 molecular weight. All of the plasma protein is in the third fraction. The membranes employed for this purpose were Visking tubing of regenerated cellulose with a pore size sufficient to allow the passage of molecules below 10000 mol. wt. For separating molecules above 1000 mol. wt. from those below, Amicon UMO-5 membrane was used.

Previously, it has been demonstrated by the lung bioassay that activity of plasma from septic patients resided primarily in the fraction of plasma containing molecular weights from 1000 to 10000. When this fraction was passed through a Sephadex G-50 column for separation into 150 tubes, optical density determinations at 280A indicated that a peak characteristically occurred in the plasma fraction (1000-10000 mol. wt.) not found in that from normal people. The patterns typical of five normal and six septic patients are illustrated in Figures 1. and 2. From these it is evident that the peak of the abnormal curve lies at tube number 85, with a mol. wt. between 4000 and 6000. Another peak is observed between tubes 27 and 35 (mol. wt. 8000-9000). Neither peak is present in the equivalent fraction from normal human plasma.

As shown in figures 3 and 4, similar chromatographic peaks are observed in the fractions of plasma from septic patients when the tubes are labelled with fluorescamine and exposed to ultraviolet light for measurement of fluorescent activity.

Recently to abtain a sufficient quantity of the chromatographic fractions for concentration, testing and possible isolation the filtered fractions of plasma (1000 to 10000 mol. wt.) from five septic patients have been pooled. When the pooled fractions are passed through the Sephadex G-50 column, the typical peaks of optical density with 280A have been found. This material is being divided into pools of tubes for further bioassay and immunoassay.

To assess the possibility that one of the peaks, characteristic of sepsis, might be a fraction of complement (conceivably C3a) activated by endotoxin through the alternate pathway, heparinized whole human blood was incubated at 37°C for one hour with endotoxin (0.03 mgm/ml.). At two different heparin concentrations (1.0 unit and 10.0 units/ml.) no abnormal peaks were found in the fractions 1000 to 10000 mol. wt. Despite this negative finding, the presence of compliment fragments in the "active" fraction (1000-10000 mol. wt.) in plasma from septic man is not ruled out since heparin can block the alternate pathway. Alternatively, insufficient lysozomal enzymes may be present in existing macrophages to activate the complement system or cleave the large complement molecule.

2.) Thin layer chromatography using silica gel G and butenol 60% with acetic acid 20% as the solvent gave bands with 65% mobility with the fraction 1000 to 10000 mol. wt. This series of bands were not found when the same fraction from normal plasma was tested. An attempt was made to reconstitute that portion which differed by scraping off the appropriate regions of a parallel unstained thin layer plate for redissolving in saline. Tests of this material in the lung bioassay system proved unsatisfactory due to the presence of an agent in the gel of the plate which caused pulmonary edema.

B. Isoelectric Focussing

Preparative: Pevicon* (a copolymer of polyvinyl chloride and polyvinyl acetate) was employed to form a 22 cm. plate. The amphocytes** were for 3 to 10 pH, and mixed with the Pevicon in a 2% solution. Current was applied at an initial voltage of 30 for an hour with 4 milliampers. Then the sample of plasma fraction 1000 to 10000 mol. wt. was introduced at the midpoint. The voltage was then raised to 200 for 18 hours. The temperature was maintained at 10^{6}C.

At the completion of the run, 1 cm. cuts were removed. The fluid content of each cut was separated by centrifugation from the Pevicon. The pH was determined in each which showed a linear gradient from 2.7 to 10.6. The fluid was assayed for biological and immunological activity. The sample from the cut at pH 5.9 when combined with the rabbit antibody to septic plasma peptides inhibited the precipitin reaction with septic plasma in the

* Mercer Chemical Company

** BioRad Lab., Richmond, Cal.

Ouchterloni plate (see below section C). This suggested that the active agent may possibly be isolated by isoelectric precipitation at pH 5.9.

C. Immunological Studies

Previously a rabbit antibody to the peptides in the fraction of plasma from septic patients containing substances from 4000 to 6000 mol. wt. was produced by coupling to CNBr-activated sepharose prior to injection into New Zealand white rabbits (7).

By means of the Ouchterloni technique a precipitin line occurred with plasma from septic patients but not with that frc normal people. As described in the Progress Report submitted a year ago good correlations were obtained with the clinical state of pulmonary shunting and the results of bioassays in the patients whose plasma was tested.

Unfortunately, the original specific antiserum was exhausted nearly a year ago. Attempts to reproduce this antiserum by injection of rabbits has met with only limited success in terms of the original clean cut results. Efforts to produce a more specific serum are being continued employing both pure bred New Zealand rabbits and guinea pigs.

Despite these frustrations, several matters of immunological importance have become evident:

1.) A precipitin reaction occurs by the Ouchterlony method when normal human plasma and normal rabbit serum are placed in

adjacent wells. This finding suggests a non-specific reaction. However, it appears to be dependant upon the presence of activated compliment, since heating both the human and rabbit serum prevents the formation of the precipitin lines.

2.) The rabbit immune serum made by injecting the sepharose-peptide complex (4000-6000 mol wt.) prepared from the plasma of septic patients, reacted with both normal and septic human plasma. However, when the antibody was absorbed by normal human plasma the reaction with normal plasma no longer took place while a weak precipitin reaction with a septic plasma remained in the Ouchterloni plate. This pattern is illustrated in Figure 5. Five normal plasmas and six of eight plasmas from septic patients reacted in such a fashion. This finding continues to support the view that a circulating polypeptide is present in the plasma of certain infected patients, which is present only in low titre or not at all in the plasma of normal people.

3.) Heating the plasma of either septic or normal patients to 56°C prevents a precipitin line with the rabbit antipeptide antibody in the Ouchterloni plate, as illustrated in Figure 5. This finding suggests that compliment is essential for the precipitin reaction in this preparation.

4.) Immunoelectrophoresis: Plasma from normal and septic patients was placed in alternate wells and subjected to electrophoresis at 60 volts and 15 m Amp. for 90 minutes. Thereafter, normal rabbit serum was placed in the trough between the reactant

wells and allowed to incubate for two days, lines of alpha 2 electrophoretic mobility were present with the normal. On the other hand, the lines formed with the septic plasma had an alpha 2 beta 1 electrophorectic mobility. Again this is further evidence of a nonspecific reaction indicating the presence of a substance different or in greater quantity in the septic plasma which causes precipitation. Possibly this may be activated compliment or a fragment thereof such as C3A or C5A.

A similar but accentuated reaction was present when rabbit anti-septic peptide antiserum was placed in the troughs. Plasma of four septic patients and three normal people all responded in this fashion. This finding further supports the view that greater quantities of certain peptides are present in the plasma of septic patients which are capable of participating in an antigen-antibody reaction leading to a specific precipitin reaction. This reaction is illustrated in Figure 6.

When rabbit anti C-3 is placed in the troughs, normal and septic plasma share an identical anodal precipitin line, while septic plasma has a cathodal line not present with the normal. This phenomenon also is illustrated in Figure 6. From this finding it is evident that the molecular components of C-3 are different in normal and septic human plasma.

IV. DISCUSSION AND SIGNIFICANCE

For several years it has been evident that patients who are severely injured and/or infected are more prone than others to pulmonary insufficiency. This complication still carries a high mortality despite the advent of respiratory support and other important therapeutic measures. Typically the pathological process starts with an interstitial pneumonitis (Phase I), and is often followed by secondary infection, bronchopneumonia (Phase II). The Phase III lesion is characterized histologically by septal edema, round cell infiltration, vascular congestion, and diffuse alveolar collapse (6).

Previous studies from many sources have demonstrated that this type of interstitial pneumonitis can be produced by serotonin, fibrinopeptides, kinins, and most importantly, by histamine (4). The pulmonary anaphylactic response is triggered via activation of compliment which induces cyclic AMP and the release of histamine from mast cells.

From the clinical studies of injured septic patients supplemented by the animal experiments, past and present, it is evident that one or more substances of low molecular weight (1000-10000) are present which injure the lung and evolve an anaphylactoid response. This phenomenon in many respects also resembles that of tissue rejection. Since only fibrinopeptides and the kinins have molecular weights above 1000, and since the role of these substances remains unproven, it has been necessary to postulate

the presence of another peptide which is capable of independantly inducing the abnormal response or which may cause the release of histamine. C3A, a known fragment of activated compliment, has a molecular weight of approximately 7000, and has the properties described above(8).

It is reasonable to suspect the presence of compliment activation in the presence of sepsis by the classical antigen-antibody pathway or by the alternate pathway which endotoxin can activate(9). Therefore, the experiments reported are the start of a series designed to demonstrate the possible role of compliment fragments as well as those of other vasoactive peptides in the plasma.

Of course, the important feature of this work is to isolate the active agent or agents by the various methods employed whether chemical or immunological, in order to gain an understanding of the mechanism so that appropriate preventive or therapeutic methods may be developed.

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TABLE 1. PULMONARY FUNCTION FOLLOWING HYPOVOLEMIA AND SEPSIS
Mean, (Range) and n = Number of Patients

	Hypovolemic (n 7)	Sepsis (n 21)	Mixed (n 12)	Normal Values
Age	57 (27-77)	49 (31-69)	43 (23-67)	
Duration of Support (days)	4 (2-8)	13 (3-47)	14 (3-43)	
Shunt per cent	39 (28-62)	34 (18-48)	42 (27-67)	8-12†
Pulmonary artery pressure (mm Hg)	14 (10-20)	24 (12-38)	24 (16-32)	15-20
Pulmonary wedge pressure (mm Hg)	6 (3-12)	12 (0-20)	14 (10-19)	4-6
Peak Inspiratory pressure (cm H_2O)	25 (17-33)	25 (17-19)	23 (15-35)	10-12
$F_{1}O_{2}$ 0.6*	55 per cent	66 per cent	66 per cent	
PEEP 10 cm H_2O*	57 per cent	62 per cent	66 per cent	

* Per cent of patients requiring oxygen fraction($F_{1}O_{2}$) 0.6 and positive end expiratory pressure (PEEP) 10 cm H_2O.

† Normal values for patients at bed rest.

TABLE 2. RESULTS OF RABBIT LUNG BIOASSAY

	VALUES AT END OF PERFUSION			
	PIP ($cm\ H_2O$)	Mean Pa Pres ($mm\ Hg$)	Edema	Reactive Response
Perfusate alone, 2 hour	(n=10) 12 ± 3	13 ± 5	0 to +	10%
Normal Human Plasma	(n=12) 15 ± 4	16 ± 5	0 to +	8%
Plasma Post Hypovolemic shock patients	(n=5) 25 ± 10	21 ± 6	0 to +++	60%
Plasma from Septic patients	(n=9) 34 ± 8	35 ± 6	++ to +++	89%

Figure 1

NORMAL C. Devit 1,000-10,000 mw brain on sephadex G-50

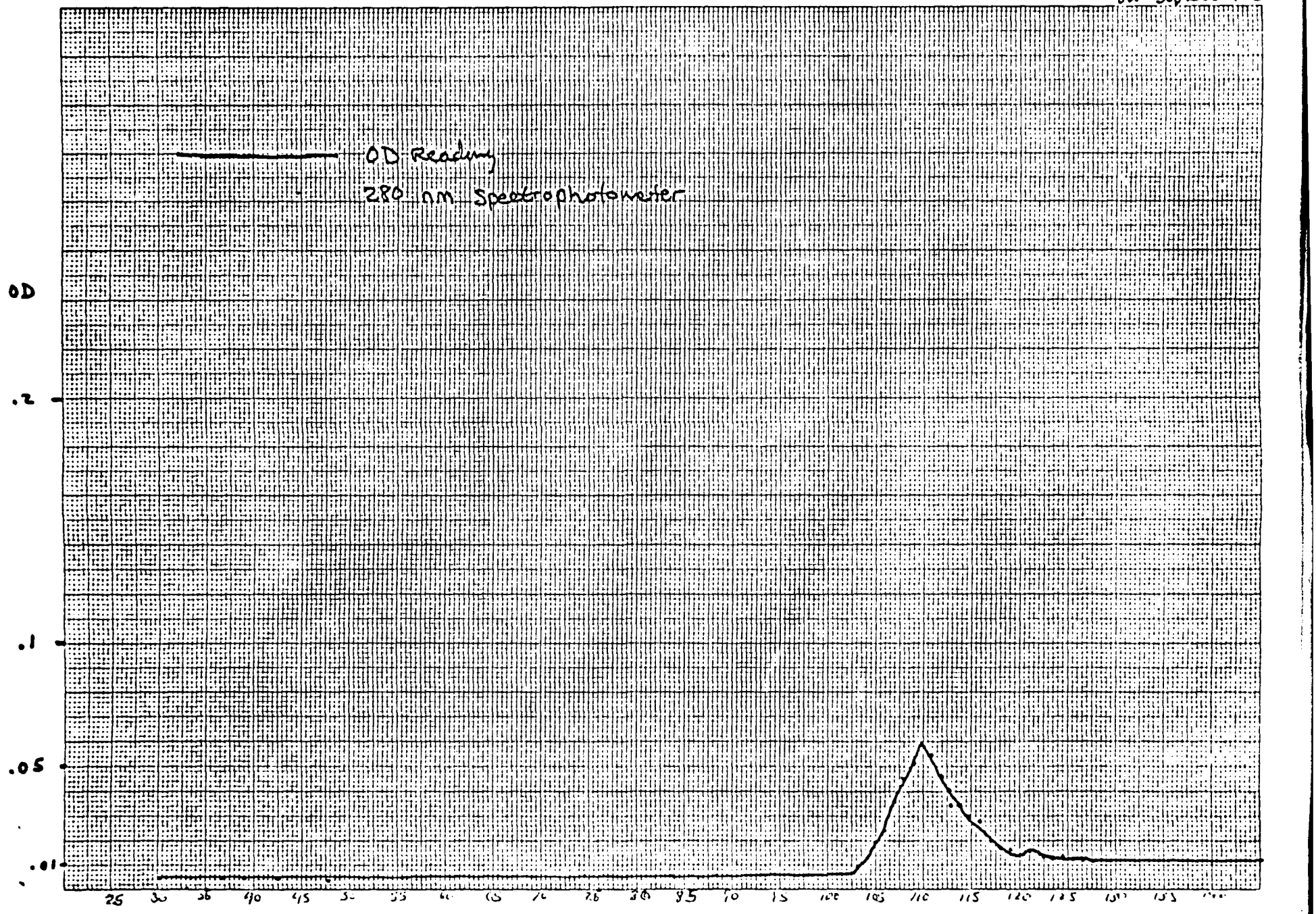

Figure 2

K•E 10 X 10 TO THE CENTIMETER 46 1510
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KEUFFEL & ESSER CO.

Sepharose G-50

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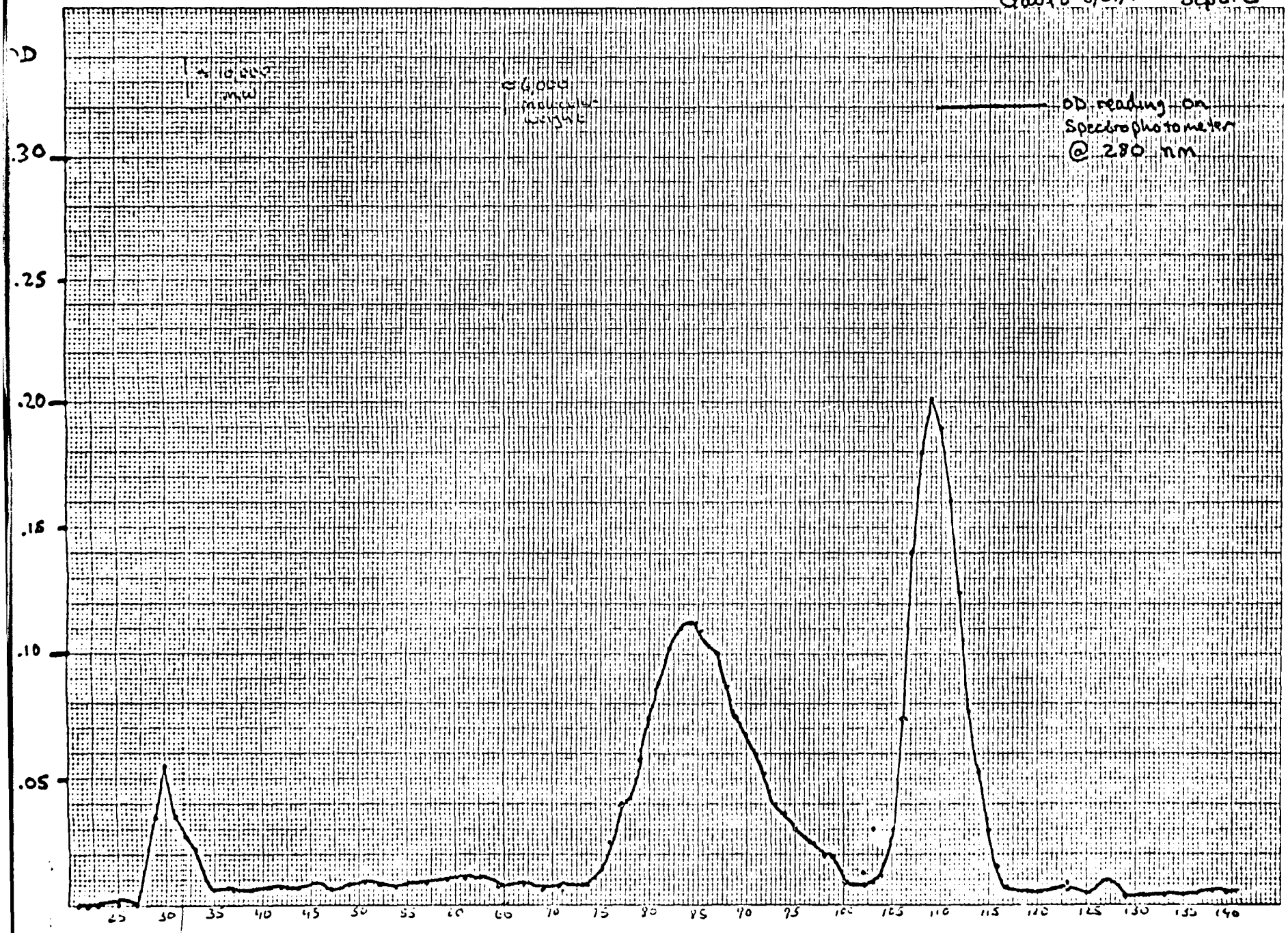

Figure 3

K&E 10 X 10 TO THE CENTIMETER 48 1510
10 X 25 CM.
KEUFFEL & ESSER CO. MADE IN U.S.A.

Fluorescamine Labeling

I.J. Newell
NIH

1,000 - 10,000 M.W on Sephadex G-50

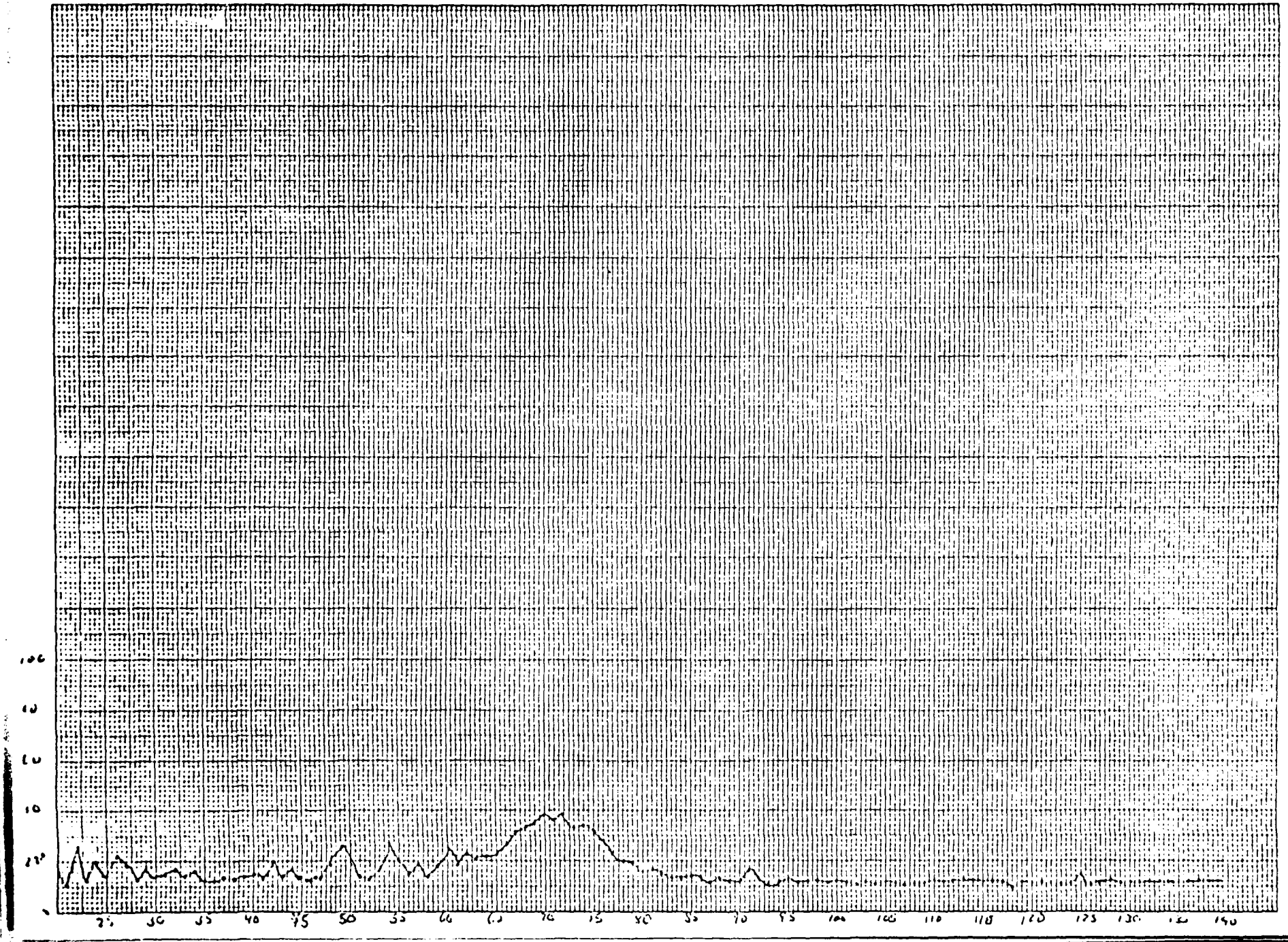

Figure 4

K&E 10 X 10 TO THE CENTIMETER 46 1510
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KEUFFEL & ESSER CO.

Flourescamine labeling

1,000 · 10,000 MW Fin.

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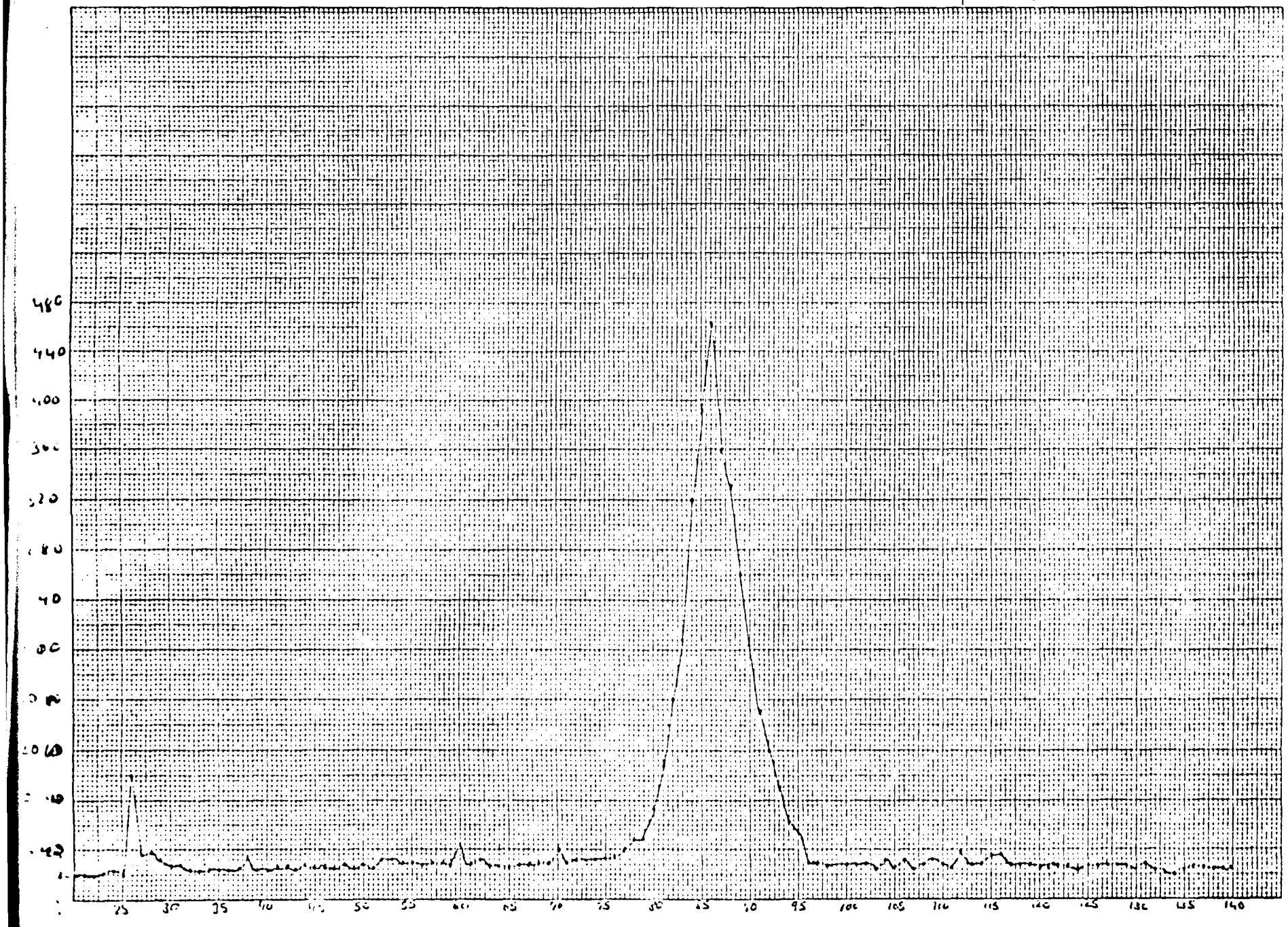


FIGURE 5.

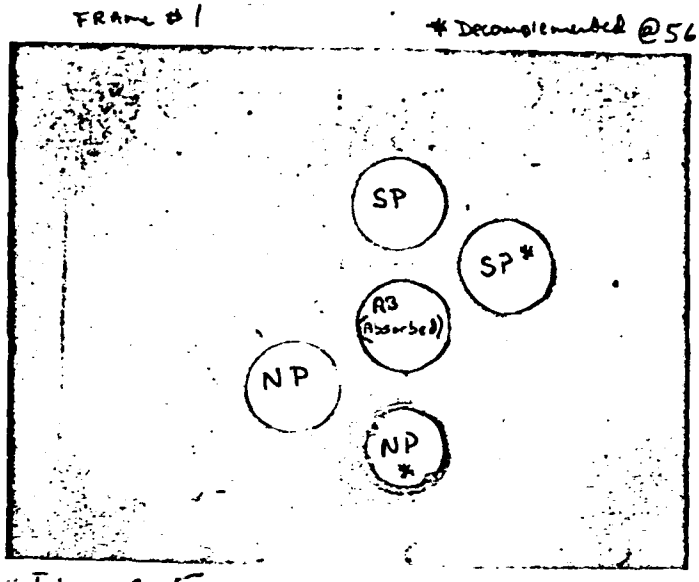


Figure 5

FIGURE 6.

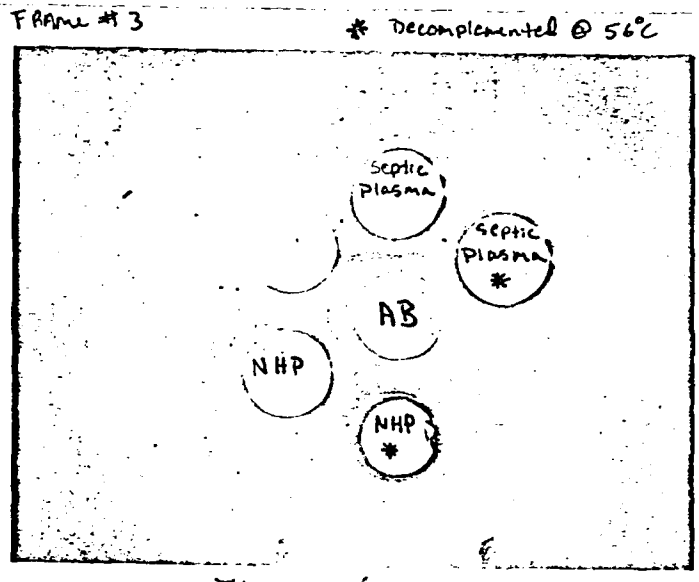
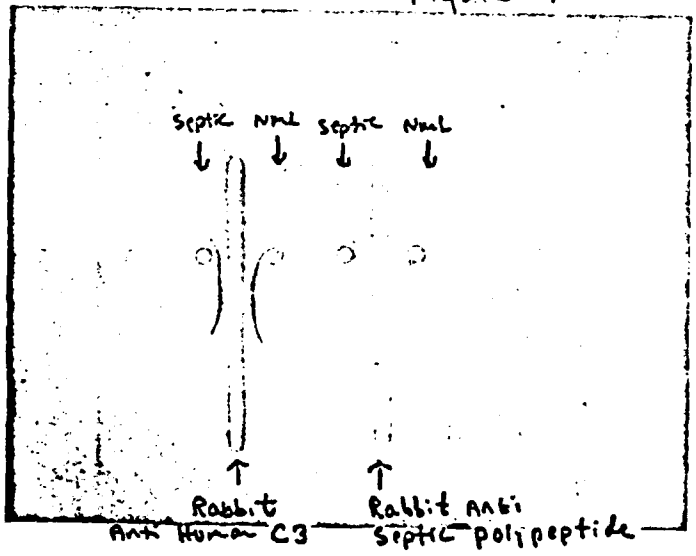


Figure 6

FIGURE 7.



TITLES

Figure 1 Normal human plasma fraction molecular weights 1000-10000, Sephadex G-50 column chromatography. Optical density of tubes at 280 A. Note isolated peak at approximately 2500 molecular weight.

Figure 2 Plasma fraction from septic patient 1000-10000 molecular weights, Sephadex G-50 column chromatography. Note abnormal peak at molecular weight 4000 to 6000 compared with Figure 1.

Figure 3 Normal plasma fraction 1000-10000 molecular weight, sephadex G-50 column chromatography labeled with fluorescamine.

Figure 4 Plasma fraction 1000-10000 molecular weight, from septic patient, Sephadex G-50 column chromatography labeled with fluorescamine. Note abnormal peak at 4000-6000 molecular weight.

Figure 5 Ouchterloni precipitin reaction of rabbit antibody to fraction 4000-6000 mol. wt. of septic plasma absorbed with normal plasma, against only plasma from septic patient but not septic plasma decomplimented by heat.

Figure 6 Same as Figure 5 except that peptide antibody is not absorbed. Note reaction with both normal and septic plasma.

Figure 7 Immunoelectrophoresis of normal and septic plasma against rabbit anti human C3 and rabbit anti septic polypeptide (mol. wt. 4000-6000) showing beta 2 activity in both antibodies. This suggests the presence of a fragment such as C3A in septic plasma.

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